DROSOPHILA CLIPPED *FRT* (cFRT) CHROMOSOME INSENSITIVE TO *P* TRANSPOSASE, GENERATING METHOD THEREOF, AND APPLICATION THEREOF

FIELD OF THE INVENTION

[0001]   The present invention is related to a *Drosophila* clipped *FRT* (cFRT) chromosome, and more particularly, to a *Drosophila* clipped *FRT* (cFRT) chromosome insensitive to *P* transposase but remaining functional to yeast site-specific flippase recombinase (FLP), the generating method thereof, and the application thereof.

BACKGROUND OF THE INVENTION

[0002]   The human diseases, the screening of the pathogenic genes and the functional research thereof are always important research fields. However, due to the systematic limitation, the possible living mechanisms are understood mostly through animal models which are composed by manipulated genes. For hundreds of years, *Drosophila Melanogaster* have been used as the basic source material for teaching and genetics study. In the last few decades, since the molecular biology method was brought into this field, the research of *Drosophila* molecular developmental genetics has entered into a new stage. The biological mechanism of *Drosophila* has the feature of evolutional conservation. Therefore, from *Drosophila* study, people understand more about that many human disease gene and cancer-related gene are derived from the gene mutation which controls the developmental mechanism. For example, the initiative mechanism of colon cancer and its complex working process are understood by studying the related genes of Wingless pathway which drives the development of abdominal segment formation in *Drosophila* embryo. Therefore, the main

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future studying direction of the present invention is doing the searching and studying of the specific functional genes in *Drosophila* genome based on the technique of the present invention. The *Drosophila* model will then be the base of searching the homologous genes of the human disease and pathogenic genes for the further study of human genetic mechanism.

[0003]   In the present time, as the structure genomic projects in model organisms are completed, how to decipher the flood of raw DNA sequences data in understanding gene function *in vivo* will be one of the major tasks for biology-related researchers. Different genomic strategies for defining and dissecting developmental and physiological pathways have been approached. The summit of these approaches is the systematic genomic screening of a specific functional trait using DNA tags such as P-transposon or retrovirus as mutagenesis agents. The designed transposon, as a mobile element, can be used to rapidly obtain cellular DNA sequence nearby the genetic mutation. The cellular DNA sequences can be obtained from the P-transposon-induced mutated genes loci by the direct use of inverse PCR (IPCR) or plasmid rescue methods. By such operating way, the inefficient and labor-intensive drill of cloning sequences for obtaining the junction sequence between the host and the mobile element can be circumvented. While retrovirus was used to mutate leukemia-causing genes in mouse (Li et al., 1999, Nature Genetics 23, 348), several hundred integration sites were cloned and characterized followed by high-throughput sequencing, data analysis and refined genetic mapping. Similarly, a global transposon mutagenesis in Mycoplasma allowed the question of the number of essential genes in a minimal genome to be answered (Hutchison, et al., 1999, Science 286, 2165). Therefore, by combining with emerging genomic

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tools, such systems in model organisms will indeed dramatically accelerate the pace of discovery in human disease-genes and cancer-related genes.

[0004]   For the functional study of *Drosophila* genes, a conventional approach relies on the creation of mosaic animals whereby the genotype varies in a cell-specific or tissue-specific manner. Currently, almost various techniques utilize the yeast FLP-FRT recombination system introduced into *Drosophila* (Golic and Lindquist, 1989, Cell 59, 499) to promote chromosomal site-specific exchange. This system allows the efficient recovery of homozygous patches in an otherwise heterozygous animal and thus permits a phenotypic analysis of mutant tissues.

[0005]   Different versions of the FLP-FRT (Flippase-Flippase Recombination Target sequence) system have been established for analyzing gene functions in either somatic or germline tissues. The direct mosaic productions in different somatic tissues have been established (Xu and Rubin, 1993, Development 117, 1223; Duffy et al., 1998, Development 125, 2263). In these methods, different tracing markers are used as the controls to monitor the presence of homozygous clones of genes to be studied. In addition, the FLP-DFS technique suitable for asking germline functions for loci resided in more than 95% genome has also been systematically completed (Chou and Perrimon, 1992, Genetics 131, 643; Chou et al., 1993, Development, 119, 1359 and Chou and Perrimon, 1996, Genetics 144, 1673). The FLP-DFS technique uses the X-linked germline-dependent dominant female sterile mutation ovo^{D1} as a selection marker for the detection of germline recombination events. Nevertheless, the FLP-FRT system is used to promote site-specific chromosomal exchange (Chou and Perrimon, 1992, Genetics 131, 643).

[0006]   However, the major drawback for all these FLP-FRT methods is that the mobile element such as the P-transposon can not be used directly as the mutagenesis agent to mutate the FRT chromosomes. While Δ2-3 transposase is recognizing the *P* transposon insertion as the mobilization origin, it simultaneously recognizes and transposes the *P[FRT]* insertions used in the FLP-FRT system. Under such situation, the genetic recombination cannot be proceeded due to the fact that the *P[FRT]* chromosomes are not homologous. For example, the mobilized *P[FRT]* will mostly create a non-homologous condition. However, the germline recombination of *P[FRT]-ovo^{D1}* chromosome needs the existence of the homologous *P[FRT]* chromosome when *P[FRT]-ovo^{D1}* chromosome is used for the FLP-DFS germline recombination. Therefore, the transposition of the *P[FRT]* insertion results in a non-homologous condition so that the germline recombination cannot be proceeded.

[0007]   Presently, only the EMS can be used for a full-scale genome-wide screening when using FRT chromosomes. Many interesting genes have been recovered. However, the goal to completely recover and to do molecular characterization of all interesting loci efficiently would be difficult if only the EMS is used for mutagenesis. Because the EMS produces mostly point mutations, it does not create any molecular tags on mutated genes for cloning manipulation. Consequently, the approach for identifying important genes is heavily impeded by the inefficient and labor-intensive traditional molecular cloning procedures.

[0008]   Another alternative strategy to facilitate gene cloning is to use transposition system independent of the P transposon. For example, the Hobo element system can be used to cause the gene mutation. However, the problem

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of creating a chromosomal environment where to completely avoid the P transposon system has not been possible in the *Drosophila* field. This kind of approach has never been described in the field while the versatile FLP-FRT- system has been publicized since 1989 (Golic and Lindquist, 1989, Cell 59, 499).

[0009]   Another way to overcome this is to individually recover the recombinant chromosome with an interested *P* insertion and the specific *P[FRT]*. This tedious and laborious work has been done for a collection of 496 P element-induced mutations established by the Berkeley Drosophila Genome Project (Perrimon et al., 1996, Genetics 121, 333). By using the FLP-DFS technique, 496 independent zygotic lethal mutations identified by single *P*-element mutations were tediously recombined with FRT chromosomes in order to analyze their germ-line clone phenotypes (Perrimon et al., 1996, Genetics 121, 333). Similarly, the same approach has been conducted in at least 7 labs in Europe. They collected only 700 recombinant chromosomes within at least 6 years. The strategy to perform similar recombination experiment is confronted with not only the prerequisite to know the location of the new P insertion for a successful recombinant but also the limitation of reaching a saturation screening since the recombination suppression exists in certain chromosome regions. These recombinants are too few to reach the 24,300 lethal chromosomes in order to reach a 87% saturation screening for the functional description of *Drosophila* essential genes (Spradling, 1999, Genetics 153, 135).

[0010]   In order to overcome the foresaid drawbacks, the present invention circumvents the above difficulties by constructing an advanced version of *P[FRT]* insertions on the *Drosophila* second chromosome, which allows the *P*-directed mutagenesis to achieve purposes of quick chromosome-wide screening and fast molecular cloning for the various FLP-FRT methods. Molecular

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biology technique such as inversed PCR (polymerase chain reaction) and plasmid rescue methods can be used to recover flanking genomic DNA sequences and relevant molecular properties of the genes affected by the transposon. Based on the mutated phenotypes of either germline or somatic recombinant clones produced, the biological functions can be described for the genes mutated. The integrated description of molecular natures and biological functions of *Drosophila* genes can accelerate the understanding of the functions of human gene homologues and be used as the basis for the application and development of gene medicines.

SUMMARY OF THE INVENTION

[0011]   It is an object of the present invention to provide a method for generating a *Drosophila* clipped *FRT* (cFRT) chromosome insensitive to *P* transposase but remaining functional to yeast site-specific flippase recombinase (FLP).

[0012]   It is another object of the present invention to provide a method for constructing an advanced version of the two *P[FRT]* insertions on the left and right arms of the *Drosophila* second chromosome, thereby forming the $cFRT^{2L2R}$ chromosome insensitive to *P* transposase.

[0013]   It is another object of the present invention to provide a $cFRT^{2L2R}$ chromosome, which allows the *P*-directed mutagenesis to achieve purposes of quick chromosome-wide screening and fast molecular cloning for the various FLP-FRT methods.

[0014]   It is another object of the present invention to provide a $cFRT^{2L2R}$ chromosome, which can be directly mutagenized by the P transposon and instantly followed by genetic recombination after the recovery of mutations.

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[0015]　According to the present invention, a method for generating a *Drosophila* clipped *FRT* (cFRT) chromosome insensitive to a *P* transposase but remaining functional to a yeast site-specific flippase recombinase (FLP), comprises steps of: (a) exposing a *FRT* chromosome to the *P* transposase for occurring a local and imprecise transposition, wherein the *FRT* chromosome contains a *P[FRT]* insertion with a selection marker gene, (b) screening the *P[FRT]* insertion insensitive to the *P* transposase to obtain screened products, (c) selecting candidate products from the screened products by further examinations, and (d) exposing the candidate products by the *P* transposase and selecting a desired product by the further examinations to obtain the *Drosophila* clipped *FRT* (cFRT) chromosome is insensitive to the *P* transposase but remaining functional to yeast site-specific flippase recombinase.

[0016]　In accordance with the present invention, the method further comprises a step (e) of examining the actual molecular nature of the clipped insertion by PCR (polymerase chain reaction)

[0017]　Preferably, the step (c) further comprises steps of: (c1) examining the screened products for both recombination capability and homozygous viability, and (c2) examining recombination accessibility of FRT sequences contained in a clipped *P[FRT]* insertion by the presence of the FLP to obtain the candidate products.

[0018]　Preferably, the recombination capability represents the functional activity of the clipped *P[FRT]* insertion and its homologous location relative to that of the original P[FRT] insertion.

[0019]　Preferably, the homozygous viability represents a genetic background after the chromosome's exposure to the *P* transposase.

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[0020] Preferably, the step (d) of exposing the candidate products by the *P* transposase and selecting the desired product by the further examinations is repeated at least twice.

[0021] Preferably, the *Drosophila* cFRT chromosome is an isogenized homozygous viable *Drosophila* second chromosome.

[0022] Preferably, the cFRT is formed due to a target sequence, recognized by the *P* transposase and responsible for a *P* transposase transposition, which is damaged and alternated into a type of incomplete target sequence, through one of a group consisting of: (1) missing of a P5' DNA sequence region, (2) missing of a P3' DNA sequence region, and (3) missing of DNA sequences other than those defined in item (1) and in item (2).

[0023] Preferably, the *Drosophila* cFRT chromosome remains the functional activity of the cFRT insertion for a site-specific recombination in the presence of the FLP.

[0024] Preferably, an effectiveness of the *Drosophila* cFRT chromosome is monitored by a FLP-FRT system and derived modification systems thereof.

[0025] Preferably, an effectiveness of the cFRT chromosome is monitored by molecular biology methods for the description of the cFRT DNA sequences configuration.

[0026] Preferably, the *Drosophila* cFRT chromosome remains to behave normally as a wild type chromosome feasible for various genetic manipulations.

[0027] Preferably, a clipped *P[FRT]* insertion is alternatively moved to another chromosome from the *Drosophila* clipped *FRT* (cFRT) chromosome by treating the *Drosophila* cFRT chromosome with one of mutagens and X-ray.

[0028] Preferably, the *Drosophila* cFRT chromosome is alternatively used to establish a *Drosophila* cell line based on a genetic background of the *Drosophila* cFRT chromosome.

[0029] Preferably, the *Drosophila* cFRT chromosome is mutated to obtain gene mutations for further experiment.

[0030] Preferably, a molecular information of the gene mutations is recovered by retrieving flanking DNA sequences of a clipped *P[FRT]* insertion with a molecular biology method.

[0031] Preferably, the molecular biology method includes a plasmid rescue method, a inversed PCR method and a chromosomal walking method.

[0032] Preferably, the molecular information of the gene mutations can be recovered by a related bioinformatic manipulation.

[0033] Preferably, the related bioinformatic manipulation includes blasting databank, searching gene homologues of biological organisms, analyzing comparative genomics, and analyzing phylogenic distance and relationship.

[0034] Preferably, the functional description of the gene mutations are further analyzed based on the information obtained from the molecular biology method and the related bioinformatic manipulation by using a biological technique.

[0035] Preferably, the *Drosophila* cFRT chromosome is used to study the *Drosophila* genes located on the second chromosome and their corresponding gene homologues of other biological organisms including vertebrates, invertebrates, eukaryotes and prokaryotes.

[0036] According to another aspect, a method for generating a *Drosophila* clipped FRT^{2L2R} (cFRT^{2L2R}) chromosome insensitive to a *P* transposase but remaining functional to a yeast site-specific flippase recombinase (FLP),

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comprises steps of: (a) exposing a double-*FRT* chromosome to the *P* transposase for occurring a local and imprecise transposition, wherein the double-*FRT* chromosome contains a first *P[FRT]* insertion with a first selection marker gene on one arm thereof and a second *P[FRT]* insertion with a second selection marker gene on the other arm thereof, (b) screening respectively the first *P[FRT]* insertion and the second *P[FRT]* insertion insensitive to the *P* transposase to obtain screened products, (c) selecting candidate products from the screened products by further examinations, and (d) exposing the candidate products by the *P* transposase and selecting a desired product by the further examinations to obtain the *Drosophila* clipped FRT^{2L2R} ($cFRT^{2L2R}$) chromosome insensitive to the *P* transposase but remaining functional to yeast site-specific flippase recombinase.

[0037]   In accordance with the present invention, the method further comprises a step (e) of examining the actual molecular nature of the clipped insertions by PCR.

[0038]   Preferably, the step (b) further comprises steps of: (b1) screening the first *P[FRT]* insertion insensitive to the *P* transposase subject to an immobility of the first selection marker gene, and (b2) screening the second *P[FRT]* insertion insensitive to the *P* transposase from the screened products of step (b1) subject to an immobility of the second selection marker gene.

[0039]   Preferably, the step (b) further comprises steps of: (b1') screening the second *P[FRT]* insertion insensitive to the *P* transposase subject to an immobility of the second selection marker gene, and (b2') screening said first *P[FRT]* insertion insensitive to said *P* transposase from screened products of step (b1') subject to an immobility of the first selection marker gene.

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[0040] Preferably, the step (c) further comprises steps of: (c1) examining the screened products for both recombination capability and homozygous viability, and (c2) examining recombination accessibility of FRT sequences contained in the *P[FRT]* insertion by the presence of the FLP to obtain the candidate products.

[0041] Preferably, the first selection marker is different from the second selection marker.

[0042] Preferably, the *Drosophila* clipped FRT^{2L2R} chromosome is alternatively generated from two *Drosophila* clipped *FRT* (cFRT) chromosomes ($cFRT^{2L}$ and $cFRT^{2R}$ chromosomes) by a genetic recombination method.

[0043] According to another aspect, a *Drosophila* clipped *FRT* (cFRT) chromosome, wherein the chromosome is insensitive to a *P* transposase but remains functional to a yeast site-specific flippase recombinase (FLP), comprises: a *Drosophila* second chromosome main body, and a clipped *P[FRT]* (cFRT) insertion immobilized by the *P* transposase.

[0044] In accordance with the present invention, the cFRT is formed due to a target sequence, recognized by the *P* transposase and responsible for a *P* transposase transposition, which is damaged and alternated into a type of incomplete target sequence, through one of a group consisting of: (1) missing of a P5' DNA sequence region, (2) missing of a P3' DNA sequence region, and (3) missing of DNA sequences other than those defined in item (1) and in item (2).

[0045] According to another aspect, a *Drosophila* clipped FRT^{2L2R} (cFRT^{2L2R}) chromosome, wherein the chromosome is insensitive to a *P* transposase but remains functional to a yeast site-specific flippase recombinase (FLP), comprises: a *Drosophila* second chromosome main body, and a clipped *P[FRT]* (cFRT) insertion on a right arm (cFRT^{2R}) of the *Drosophila* second

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chromosome and a clipped $P[FRT]$ (cFRT) insertion on a left arm (cFRT^{2L}) of the $Drosophila$ second chromosome, wherein both the cFRT^{2R} and the cFRT^{2L} are immobilized by the P transposase.

[0046] In accordance with the present invention, the $P[FRT]$ insertions on a left arm is inserted into the 3' end of the base T at 240696 bp of the AE003781 clone with the P3' end facing the centromere before being clipped, and said $P[FRT]$ insertion a right arm is inserted into the 3' end of the base T at 11497 bp of the AE003789 clone with the P5' end pointing toward the telomere before being clipped.

[0047] Preferably, the cFRT^{2L} is an imprecise excision caused by a removal of P5' region and most part of a selection marker gene thereon, wherein a fragment from bases 26 to around 2070 of FBtp0000348 locus is deleted.

[0048] Preferably, the cFRT^{2R} is an imprecise excision caused by a removal of most of the P5' region and one of the FRT DNA repeats, wherein a fragment from bases 10 to 2821 of FBtp0000268 locus is deleted.

[0049] The foregoing and other features and advantages of the present invention will be more clearly understood through the following descriptions with reference to the drawings, wherein:

BRIEF DESCRIPTION OF THE DRAWINGS

[0050] Fig. 1 is a diagram showing the position of the $P[FRT]$ insertion on the left arm of the FRT chromosome before being clipped according to a preferred embodiment of the present invention;

[0051] Fig. 2 is a diagram showing the $P[FRT]$ insertion on the left arm of the FRT chromosome before being clipped according to a preferred embodiment of the present invention;

[0052]   Fig. 3 is a diagram showing the *P[FRT]* insertion on the left arm of the *FRT* chromosome after being clipped according to a preferred embodiment of the present invention;

[0053]   Fig. 4 is a diagram showing the position of the *P[FRT]* insertion on the right arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention;

[0054]   Fig. 5 is a diagram showing the *P[FRT]* insertion on the right arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention; and

[0055]   Fig. 6 is a.diagram showing the *P[FRT]* insertion on the right arm of the *FRT* chromosome after being clipped according to a preferred embodiment of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0056]   The present invention will now be described more specifically with reference to the following embodiments.   An advance way is provided to circumvent the previous problem by generating a new "double *FRT*" chromosome (where the "double *FRT*" chromosome was originally generated by Chou and Perrimon, 1996) which is insensitive to *Δ2-3* transposase. On the double *FRT* chromosome, two *P[FRT]* insertions are located at the base of each chromosomal arm, as the targets for FLP recombinase. These double *P[FRT]* insertions were specifically designed to facilitate the large scale recombination clonal analysis of autosomal genes.   However, the *P* transposase target sequences 12 contained in *P[FRT]* transposon are sensitive and will respond to the presence of P-transposase in the genome designed for P transposon-directed mutagenesis. To modify the double FRT chromosomes so that they are no

longer sensitive to P-transposase will be the first step toward the strategy using the P-directed, but not the EMS, mutagenesis in genome-wide screening.

[0057]   P donor elements are frequently excised and reinserted locally (Zhang and Spradling, 1993, Genetics 133, 361). The local transposition derivatives can be the candidates for the imprecise transposition events among which the P transposase target sequences of *P[FRT]* have been impaired and become insensitive to the P-transposase activity. Experimentally, several steps need to be repeatedly operated.

[0058]   1. Double *FRT* chromosomes will be exposed to the P-transposase for local and imprecise transposition to occur.

[0059]   2. Both double *FRT* chromosomes contain one *P[FRT]* insertion with *mini-white*$^+$ gene on one arm and another *P[FRT]* with *rosy*$^+$ gene on the other arm, wherein both the *mini-white*$^+$ gene and the *rosy*$^+$ gene are selection marker genes. The imprecise transposition event will be followed by the mosaic white eyes in one arm or the loss of *rosy*$^+$ gene in another arm.

[0060]   3. The two *P[FRT]* insertions insensitive to P-transposase can be first screened subject to an immobility of *miniwhite* marker. The mosaic white eyes will reflect the persistent transposition of *P[FRT]* and the non-mosaic white-plus eyes will represent the insensitivity to the P-transposase.

[0061]   4. The *FRT* derivatives with non-mosaic white-plus eyes will be the source for the search for the loss of *rosy*$^+$ gene in another arm. Derivatives fulfilled with both criteria will be examined for both the recombination capability, which represents the functional activity of *P[FRT]* insertion and its homologous location in compared with original P[FRT] insertion, and the homozygous viability, which represents the genetic background which is not detectably changed after its exposure to the P transposase.

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[0062]   5. The recombination accessibility of FRT sequences will be examined by the presence of FLP. Either germline or somatic recombination clone occurrence can be used to estimate the recombination event.

[0063]   6. The stabilized or clipped *P[FRT]* insertion candidates will be challenged by the P-transposase for the second time.

[0064]   7. The FRT sequences insensitive to P-transposase, the recombination accessibility of FRT and the homozygous viability will be examined again.

[0065]   8. The steps 6 and 7 will be cycled for at least twice to ensure the transposition insensitivity and recombination accessibility and homozygous viability of the double *FRT* chromosomes.

[0066]   9. The actual molecular nature of the clipped insertions can be examined by PCR using primer sets encompassing the target sequences of the P transposase.

[0067]   After three rounds of P-transposase treatment, one derivative of the original FRT^{2L2R} chromosome was selected as the modified and advanced $cFRT^{2L2R}$ chromosome which is insensitive to P-transposase challenges. However, the $cFRT^{2L2R}$ chromosome is functional for the occurring of the recombination on both 2L and 2R arm when using the original homologous *P[FRT]* insertions for germline clonal analysis. This homozygous viable $cFRT^{2L2R}$ chromosome is absent of both P5' inverted repeat sequences required for P transposition at both 2L and 2R *P[FRT]* insertions. These damages may cause this chromosome insensitive to P-transposase. In other words, the *Drosophila* $cFRT^{2L2R}$ chromosome contains a $cFRT^{2L}$ which is a clipped *P[FRT]* insertion on the left arm, and a $cFRT^{2R}$ which is a clipped *P[FRT]* insertion on

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the right arm. The cFRT^{2L} and the cFRT^{2R} are insensitive to P transposase and can not be mobilized by P transposase.

[0068] In addition, in another embodiment of the present invention, based on the imprecise transposition and local transposition, the original double FRT^{2L2R} chromosome was challenged with serial rounds of $\Delta2$-3 transposase treatment. This chromosome contains the $P[FRT40A]$ insertion carrying $rosy^+$ eyecolor marker and the $P[FRT42B]$ insertion carrying $miniwhite^+$ eyecolor marker. Mosaic miniwhite$^+$ eyecolor production represents the responsiveness of the P[FRT] insertion to the P transposase. After the first two rounds of challenge, derivative chromosomes with the loss of both mosaic miniwhite$^+$ and rosy$^+$ eyecolor markers are selected for further treatment as long as they are homozygously viable and functional for germline clone production based on the FLP-DFS technique. Then, further rounds of treatment will select those derivatives that produce offspring all behaved similarly to parent chromosomes in terms of being homozygous viable with compatible recombination frequency. One candidate meeting the criteria has been shown to be absent of P5' inverted repeat sequences required for P transposition in both 2L and 2R P[FRT] insertions.

[0069] This unique chromosome is deemed the "$cFRT^{2L2R}$", which particularly combines the P transposon and the FLP-FRT site-specific recombination system as an integrated one for almost 40% of $Drosophila$ genome. It allows the direct use of P transposon as mutagen for the entry of molecular cloning drill and simultaneously the functional analysis of the genes disrupted by P insertions. This is first examined by using the FLP-DFS technique for a small scale of germline clone (GLC) screen for 36 P-induced

essential loci. Specific maternal effect phenotypes can be simultaneously characterized with respect to their GLC phenotypes and their molecular properties be assigned according to the flanking genomic DNA sequences recovered by plasmid rescue and inverted PCR methods for the *P* insertions.

[0070] In order to describe the present invention in a greater detail, the material and method used in the embodiments are disclosed below. Flies used here were raised on standard *Drosophila* media at 25 ℃ unless indicated. Description of balancers and mutations can be found in Lindsley and Zimm (1992). The jumpstarter stock strain utilized here is $\Delta2$-$3(P[ry+;\Delta2$-$3]99B)$that carries a defective P element on the third chromosome at 99B which constitutively express high levels of transposase but can not itself transpose (Robertson et al. 1988). The *yw*; $\Delta2$-3, *Sb/TM6, Ubx* stock was obtained from the Bloomington Stock Center.

[0071] The autosomal FLP-DFS technique is used for the production of germ line mosaics. To generate homozygous GLCs, females were crossed with males of genotype $ywFLP^{12}/Y$; $CyO/P[ovo^{D1}]^{2L}FRT^{2L}$ or $ywFLP^{12}/Y$; $CyO/P[ovo^{D1}]^{2R}FRT^{2R}$. These males were generated by crossing females from the $ywFLP^{12}$; CyO/Sco with the appropriate $P[ovo^{D1}]FRT$ males. Females of appropriate genotypes were allowed to lay eggs for 1 day in vials and the progeny heat shocked once for 2 hrs at 37℃ in a circulating water bath over a period of 2 days when they reached late L2 to L3 larval stages as described (Chou and Perrimon, 1996). Subsequently, females of the appropriate genotype were analyzed for the presence of GLC ovaries.

[0072]   Germline clones in females heterozygous for ovo^{D1} were identified by ovary dissection. Females were dissected four days following eclosion. Since the ovo^{D1} mutation perturbs early oogenesis, a germline clone is identified by the presence of vitellogenic egg chambers. The percentage of mosaic ovaries ($\%MO$) is calculated by dividing the number of ovaries carrying vitellogenic egg chambers with the total number of ovaries examined.

[0073]   Chromosomes derived from FRT^{2L2R} were used as genomic DNA source for PCR examination. Single fly of various genotypes were used for the DNA isolation. PCR reaction was performed using touch-down melting temperature from 65-55°C. The presence of P5' and P3' regions were detected by standard PCR reaction. The primer sets used are listed below.

IR (1-20)	CATGATGAAATAACATAAGG
P5' sense (26-45)	CCGTCGAAAGCCGAAGCTTA
P5'antisense (264-232)	CCCAAGGCTCTGCTCCCACAATT
P3' sense (2756-2775)	AAACCCCACGGACATGCTAA,
P3' antisense (2878-2839)	CGGCAAGAGACATCCACTTA.

[0074]   The information of the P[lArB] element insertions is examined by inverse PCR. A sample of genomic DNA equivalent to 5-6 flies was digested with restriction enzymes Pvu II, Sau 3AI, or HinP1I separately. The digested DNA was religated with T4 DNA ligase at low DNA concentration about 0.5 ng/µl DNA. The ligation mix was extracted with phenol-chloroform, precipitated with ethanol and finally dissolved in 20µl distilled water. 2µl of each product was used for PCR amplification. Plac1 and Splac2 primers (designed by BDGP) were used for first P insertion 5' end PCR amplification for 25 cycles. The amplified products were diluted 50-fold, and 1µl of the

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diluted product was used for a second round of PCR for 30 cycles using two nested primers, sp1 and Splac2 (designed by BDGP). Subsequently, the amplified product was sequenced directly using a nested primer, Sp1 or Splac2.

[0075] The information of the FRT^{2L} and FRT^{2R} insertions is examined by inverse PCR. All the reaction conditions are similar to the above description, except primers and restriction enzymes used. For FRT^{2L}, the Sau3A enzyme was used to digest genomic DNA, and Pry1 and Pry2 primers (designed by BDGP) were used for IPCR, and Sp3 (designed by BDGP) primer was used for DNA sequencing. For FRT^{2R}, the HipI enzyme was used to digest genomic DNA, and Pry1 and Pry2 primers (designed by BDGP) were used for IPCR, and Pry1 (designed by BDGP) primer was used for DNA sequencing. The primer sequences are listed below.

Plac1	CAC CCA AGG CTC TGC TCC CAC AAT
Pry1	CCT TAG CAT GTC CGT GGG GTT TGA AT
Pry2	CTT GCC GAC GGG ACC ACC TTA TGT TAT T
Splac2	GAA TTC ACT GGC CGT CGT TTT ACA A
Sp1	ACA CAA CCT TTC CTC TCA ACA A
Sp3	GAG TAC GCA AAG CTT TAA CTA TGT

[0076] As to the respect of the plasmid rescue method, a sample of genomic DNA equivalent to 5-6 flies was digested with appropriate restriction enzymes separately (e.g. Xbal and Sac II for PZ line, Hind III and Kpn I for *P[lArB]* line), then ligated with T4 DNA ligase to circularize the restriction fragments. The ligation mix was precipitated with ethanol and dissolved in 2µl distilled water. 1µl of the ligation DNA was added to 20µl of the competent cells (DH10α). The competent cells were transformed by electroporation. The resulting colonies were pick and plasmid was extracted by using mini-

preparation method. The plasmid was cut by single cut (e.g. Hind III for Plar B line) and double cut (e.g. Hind III and Sac II for Plar B line) to check size in agarose gel. Finally, the colony with correct size plasmid was incubated and sequenced using primer pry2 (designed by BDGP).

[0077] As to the respect of the method of Intrachromosomal flip-out experiment, the intrachromosomal flip-out experiment described previously (Golic and Linquist, 1989) is followed to removed miniwhite+ marker in $cFRT^{2L2R}$ chromosome. The 24-36hours offspring from the w/Y; $cFRT^{2L2R}/cFRT^{2L2R}$ males crossed with $ywFLP^{12}$; CyO/Sco females were heat shock treated for 2 hrs at 37 °C. The adult progeny were crossed with w/w; CyO/Sco females to examine the possible presence of white eyecolor offspring. The removal of miniwhite+ marker will produce white eyecolor progeny as the result of intrachromosomal FRT-FRT recombination.

[0078] As to the EMS mutagenesis method, 70-90 isogenized $cFRT^{2L2R}$ males were fed with a mixture of 5mM EMS and 1% glucose solution for one day. These EMS-treated flies were mated to w/w; Sco/CyO females. White-eye male progenies which indicate that the loss-of-function of the *miniwhite* gene on the $cFRT^{2R}$ sequence were maintained for further analyses.

[0079] The DNA configuration of *clipped FRT* chromosome is determined by PCR. After IPCR and plasmid rescue manipulations, the *P[FRT40A]* and the *P[FRT42B]* insertion points were determined (the results are shown in the following embodiments). The molecular nature of the *clipped P[hsneo>>, ry⁺, FRT]⁴⁰ᴬ* and *clipped P[>wʰˢ>, FRT]⁴²ᴮ* of *hL18C1* were further defined by different primer sets to refine the configuration of the modified *P[FRT]* insertions.

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[0080]   The primers used are defined by the 5' to 3' sequence base pair numbered in the FBtp0000348 locus, that is the $P[hsneo>>, ry^+, FRT]$ element, and AE003781 genomic clones for *clipped* $P[hsneo>>, ry^+, FRT]^{40A}$, and in the FBtp0000268 locus, that is the $P[>w^{hs}>, FRT]$ element, and AE003789 genomic clones for *clipped* $P[>w^{hs}>, FRT]^{42B}$.

Primers used for *clipped* $P[hsneo>>, ry^+, FRT]^{40A}$ PCR reactions

40SE	CGACGAGTTGCTTCTCCCACA	240516-240536 in AE003781 clone
40AS	GTTTCCCTCGCACTGCTATTT	240952-240932 in AE003781 clone
5'SE	CCGTCGAAAGCCGAAGCTTA	26-45 in FBtp0000348 locus
5'AS	CCCAAGGCTCTGCTCCCACAATT	254-232 in FBtp0000348 locus
ry1	CGCACGGTTTCAATCACA	948-930 in FBtp0000348 locus
ry2	GGTTACGAGGCAGCAGTTCTA	2070-2050 in FBtp0000348 locus
ry3	AACGCCCACTTCCGTATTGC	4035-4016 in FBtp0000348 locus
ry4	AATCCTGGTGCTTGCTTTCCT	6092-6072 in FBtp0000348 locus
Hsp	GTAGGTCATTTGTTTGGCA	8028-8046 in FBtp0000348 locus
neo	CTGATGCCGCCGTGTTC	8587-8603 in FBtp0000348 locus
FRTf	CCCCGCATGGAATGGGATAAT	9609-9629 and 10326-10346 in FBtp0000348 locus
FRTr	AGTCCGGTGCGTTTTT	9948-9933 and 10665-10650 in FBtp0000348 locus
3'SE	AAACCCCACGGACATGCTAA,	14861-14880 in FBtp0000348 locus
3'AS	CGGCAAGAGACATCCACTTA.	14993-14974 in FBtp0000348 locus

Primers used for *clipped* $P[>w^{hs}>, FRT]^{42B}$ PCR reactions are listed below

42SE	TGCTCGCTTGGATGAAC	11032-11047 in AE003789 clone
42AS	AGTGGAGTGGGAGTGGA	11600-11584 in AE003789 clone
5'SE	CCGTCGAAAGCCGAAGCTTA	26-45 in FBtp0000268 locus
5'AS	CCCAAGGCTCTGCTCCCACAATT	254-232 in FBtp0000268 locus
FRTf	CCCCGCATGGAATGGGATAAT	2549-2529 and 7937-7917 in FBtp0000268 locus
FRTr	AGTCCGGTGCGTTTTT	2210-2225 and 7598-7613 in

		FBtp0000268 locus
3'SE	AAACCCCACGGACATGCTAA	15101-15120 in FBtp0000268 locus
3'AS	CGGCAAGAGACATCCACTTA	15214-15233 in FBtp0000268 locus

[0081] In order to describe the present invention in a greater detail, the experimental data are disclosed below. The FRT^{2L2R} chromosome contains $P[hsneo>>, ry+, FRT]$ insertion on 40A and $P[>w^{hs}>, FRT]$ insertion on 42B (Chou and Perrimon, 1996). The treatment of this double $P[FRT]$ chromosome with $\Delta 2\text{-}3$ transposase is expected to produce mosaic miniwhite[+] eyecolor after the first round treatment and to recover non-miniwhite-mosaic eye color phenotype after the second challenge if the $P[>w^{hs}>, FRT]$ transposon is not sensitive to $\Delta 2\text{-}3$ transposase (as shown below).

$\Delta 2\text{-}3$ transposase treatment for the recovery of the loss of mosaic miniwhite eyecolor production

1. $w/Y;\ FRT^{2L2R}\ /\ FRT^{2L2R}\ $ X $yw/yw;\ \Delta 2\text{-}3,\ Sb/TM6,\ Ubx$

2. $yw/Y;\ FRT^{2L2R}\ /+;\ \Delta 2\text{-}3,\ Sb/+$ X $yw/yw;\ \Delta 2\text{-}3,\ Sb/TM6,\ Ubx$

3. $yw/Y;\ (FRT^{2L2R}\ )^c/+;\ \Delta 2\text{-}3,\ Sb/+\ or\ TM6,Ubx$ X $w/w;\ CyO/Sco$

4. Pick up males without mosaic miniwhite eyecolor and set up individual lines.

[0082] After two rounds of treatments, it is also expected that the loss of $ry+$ eyecolor will represent the imprecise excision of $P[hsneo>>, ry+, FRT]$ transposon(as shown below).

Selection of $(FRT^{2L2R})^c$ chromosome with the loss of rosy+ eyecolor

1. w/Y; $(FRT^{2L2R})^c$ / $(FRT^{2L2R})^c$ X $+/BsY$; Sp/CyO; $MKRS/TM2,ry$

2. $+/Y$; $(FRT^{2L2R})^c$ $/CyO$; $MKRS/+$ X ry/ry

3. $+/Y$; $(FRT^{2L2R})^c$ $/+$; $MKRS,ry/ry$

4. search for $(FRT^{2L2R})^c$ chromosome which is lost of $ry+$ eyecolor

[0083] From 427 independent FRT^{2L2R} derived chromosomes which displayed nonmosaic miniwhite eyecolor, 107 ry eyecolor lines are obtained. After GLC analysis of these lines, three lines are capable of performing recombination on both 2L and 2R arms and 6 lines on 2R arm only. Others displayed destroyed or very low frequency of recombination ability representing the mobilized $P[FRT]$ producing a damaged FRT DNA sequences or, alternatively, a non-homologous condition with respect to $P[FRT]ovo^{D1}$ chromosome used.

[0084] The three lines, $hL18$, $hL92$, and $hL97$ are homozygously viable with comfortable ratio. Further treatments were focused on these three candidates.

[0085] Further treatments of chromosomes selected from previous challenges are examined by two criteria, homozygous viability and germ-line clone (GLC) recombination frequency. The lethality produced during transposase challenge would represent either the accumulation of mutations or the damage of loci flanking the previous P insertions caused by mobilization. GLC recombination will provide further information regarding the presence of FRT sequences, and possibly the degree of damage received.

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[0086]   The further treatments of the *hL18*, *hL92*, and *hL97* lines removed *hL92* as the possible candidate since 15 lines originated from *hL92* all displayed homozygous lethality. This clearly showed that *hL92* is very sensitive to the P transposase.

[0087]   After the third round treatment, *hL97* derivatives showed weak sensitivity to the P transposase since different lines displayed fluctuated homozygous viability (as shown in Table 1A).

Table 1A. The third round

LINE	H.V	%
hL97C1	2/34	6
hL97C1'	2/24	8
hL97C3	15/48	31
hL97C3'	10/30	33
hL97C3''	5/15	33
hL97C4	0/21	0
hL97C4'	0/33	0
hL97C5	13/60	17
hL97C5'	4/29	14
hL97C6	9/41	22
hL97C14	5/20	25
hL97C16	0/28	0
hL97C16'	0/30	0

HV: Homozygous viability is the number of homozygous flies divided by the number of total flies (homozygous + heterozygous). In theory, 33% would represent an expected homozygously viable rate.

GLC and %MO: Germ-line clonal frequency is calculated as the percentage of mosaic ovaries (%MO) of the females dissected. %MO=[number of ovaries with GLC clones/ total number of ovaries examined] x 100%.

[0088]   *hL97C3''* line was treated further since homozygously viable stock can be established. After fourth round, homozygous viability displayed smaller degree of fluctuation compared with the result from the third round (as shown in Table 1B)

Table 1 B. The fourth round

LINE	H.V.	%	2LGLC	%MO	2RGLC	%MO
hL97C3"C1	36/161	22	33/40	83		
hL97C3"C3	53/182	29				
hL97C3"C4	46/158	29				
hL97C3"C7	139/446	31	129/194	67	87/100	87
hL97C3"C8	29/105	28				
hL97C3"C10	106/316	34				
hL97C3"C12	103/445	23	·26/30	87	14/14	100
hL97C3"C14	12/90	8	54/84	64	41/48	85
hL97C3"C15	130/420	31	25/46	54	26/36	72
hL97C3"C16	6/45	13				
hL97C3"C18	18/111	16				
hL97C3"C20	70/351	20	40/64	63	71/98	72
hL97C3"C21	47/326	14				

[0089]   However, only 8 out of 13 lines can be established as homozygously viable stocks. The result obtained from fifth round of treatment (as shown in Table 1C) suggests that few *hL97C3''* derivatives can tolerate the transposase challenges.

Table 1C. The fifth round

LINE	HV	%	2L GLC	%MO	2RGLC	%MO
hL97C3"C1C1	39/174	22	50/78	64	17/26	65
hL97C3"C3C1	19/93	20	38/66	58	22/30	73
hL97C3"C3C2	25/107	23	19/28	68	16/20	80

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hL97C3"C7C2	18/48	38				
hL97C3"C7C3	36/164	28	26/38	68	46/58	79
hL97C3"C7C5	13/48	27				
hL97C3"C15C1	40/81	49				

[0090]   After the third round of transposase treatment, 2 independent derivatives, *hL18c1* and *hL18c3* were picked up for further treatment since both are homozygously viable and have 55 to 77% recombination frequency on both 2L and 2R arms (data not shown). After the fourth round, 11 derivatives from *hL18c1* all display expected homozygous viability ratio and constant recombination frequency ranging from 55 to 80 (as shown in Table 2A).

Table 2A. *hL18C1*

LINE	HV	%	2L GLC	%MO	2R GLC	%MO
hL18c1c1	95/280	32	43/64	67	41/58	71
hL18c1c2	88/248	35	35/56	63	38/56	68
hL18c1c3	90/229	39	45/68	66	26/34	76
hL18c1c4	107/231	46	83/98	85	40/52	77
hL18c1c5	89/231	38	66/82	80	28/50	56
hL18c1c6	97/232	42	76/110	69	30/54	56
hL18c1c7	79/226	35	108/182	59	50/66	76
hL18c1c8	82/232	35	57/90	63	25/42	60
hL18c1c9	116/282	41	74/118	63	5/12	42
hL18c1c10	89/251	35	60/90	67	30/40	75
hL18c1c11	104/302	34	43/68	63	29/52	56

[0091]   These results meet the criteria as the *hL18c1* chromosome is devoid of transposase sensitivity. Nevertheless, it is still able to perform the FLP-FRT recombination with a comfortable frequency.

[0092]   1 out of 11 *hL18C3* derived lines showed homozygous lethality. In comparison with *hL18c1*, this suggests that the *hL18c3* chromosome is

remaining sensitive to the P transposase in a low frequency manner (as shown in Table 2B).

Table 2B. *hL18C3*

LINE	HV	%	2LGLC	%MO	2RGLC	%MO
hL18c3c1	76/265	29	71/94	76	51/74	69
hL18c3c2	120/301	40	51/82	62	26/58	45
hL18c3c3	83/218	38	77/110	70	32/56	57
hL18c3c4	101/255	40	139/196	71	49/56	88
hL18c3c5	0/120	0				
hL18c3c6	144/293	49	27/58	47	37/62	60
hL18c3c7	97/262	37	41/74	55	22/50	44
hL18c3c8	75/231	32	29/48	60	22/40	55
hL18c3c9	95/298	32	30/58	52	30/50	60
hL18c3c10	85/245	35	60/82	73	8/16	50
hL18c3c11	101/240	42	23/56	41	41/64	64

[0093]   The molecular nature of *hL18C1* was further defined first by IPCR to define the loci of *P[hsneo>>, ry$^+$, FRT]^{40A}* and *P[>w^{hs}>, FRT]^{42B}*, and second by different primer sets to refine the configuration of the modified *P[FRT]* insertions.

[0094]   Please refer to Fig. 1, which is a diagram showing the position of the *P[FRT]* insertion on the left arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention. Originally, P[hsneo>>, ry+, FRT]40A is inserted into the 3' end to the base T at 240696 bp of the AE003781 clone with the P3' end facing centromere. Please refer to Fig. 2, which is a diagram showing the *P[FRT]* insertion on the left arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention. The *P[FRT]* insertion on the left arm

includes P5' region (1-157 bp, green color), pUC8 (547-704 bp and 10892-13391 bp, white color), rosy (922-3164 bp and 3171-7992 bp, red color), Hsp70Bb (8003-8449 bp and 13412-13858 bp, purple color), neoR (8463-9457 bp and 13872-14866 bp, blue color), FRT (9468-10174 bp and 10185-10891 bp, yellow color), and P3' region (15041-15262 bp, pink color). Please refer to Fig. 3, which is a diagram showing the P[FRT] insertion on the left arm of the FRT chromosome after being clipped according to a preferred embodiment of the present invention. In clipped P[hsneo>>, ry+, FRT]40A, imprecise excision caused the removal of P5' region and most part of the rosy+ DNA segment, e.g. fragment from bases 26 to around 2070 of FBtp0000348 locus were known being deleted.

[0095]   Please refer to Fig. 4, which is a diagram showing the position of the P[FRT] insertion on the right arm of the FRT chromosome before being clipped according to a preferred embodiment of the present invention. P[>whs>, FRT]42B is originally inserted into the 3' end to the base T at 11497 bp of the AE003789 clone with the P5' end pointing toward telomere. Please refer to Fig. 5, which is a diagram showing the P[FRT] insertion on the right arm of the FRT chromosome before being clipped according to a preferred embodiment of the present invention. The P[FRT] insertion on the right arm includes P5' region (1-587 bp, green color), FRT (594-2690 bp and 7372-8078 bp, yellow color), mini-white (2728-2937 bp, 2944-6413 bp and 6434-6966 bp, white color), Hsp70A (6981-7349 bp, purple color), and P3' region (15041-15262 bp, pink color). Please refer to Fig. 6, which is a diagram showing the P[FRT] insertion on the right arm of the FRT chromosome after being clipped according to a preferred embodiment of the present invention. Most of the most P5' region and one of the FRT DNA repeats, e.g. bases 10 to 2821 of FBtp0000268 locus, were

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deleted in the clipped P[>whs>, FRT]42B during this trimming action. The clipped $P[FRT]$ insertion on the right arm includes P5' region (1-9 bp, green color), FRT (4560-5266 bp, yellow color), mini-white (10-125 bp, 132-3601 bp and 3622-4154 bp, white color), Hsp70A (4169-4537 bp, purple color), and P3' region (5297-5529 bp, pink color).

[0096] The P transposase recognizes the 5' and 3' ends including inverted repeat and other internal sequences as targets of transposase for P transposition (Kaufman et al., 1989). The configuration suggests that both *clipped* $P[hsneo>>$, ry^+, $FRT]^{40A}$ and *clipped* $P[>w^{hs}>$, $FRT]^{42B}$ in this new chromosome are lacking of the P5' region. Nevertheless, FRT DNA sequences remain and are still fully functional for FRT-based site-specific recombination. Due to the clipping away of the two $P5'$ substrate regions, $hL18c1$ are renamed the "*clipped* FRT^{2L2R} ($cFRT^{2L2R}$)" chromosome for the *clipped* $P[FRT]^{2L}$ • *clipped* $P[FRT]^{2R}$ transposon insertions on the second chromosome.

[0097] $P[>w^{hs}>$, $FRT]$ insertion on 42B provides the miniwhite marker of FRT^{2L2R} chromosome. The miniwhite marker can be removed by intrachromosomal recombination between the two FRT DNA sequences (Golic and Lindquist, 1989). To perform P-directed mutagenesis scheme using P[lacW] instead of P[ry+] transposon, the miniwhite marker from $cFRT^{2L2R}$ chromosome was attempted to be removed. In contrast to positive result when $hL18$ is treated, the miniwhite marker cannot be removed from $cFRT$ chromosome. At least five trials were done by different people showing the same result. Though the clipped $P[>w^{hs}>$, $FRT]$ insertion on 42B can accept the $P[>w^{hs}>$, $FRT]$ insertion on the homologous chromosome for interchromosomal recombination, it can not perform the intrachromosomal recombination.

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[0098]   To search for $cFRT^{2L2R}$ chromosome with *white-minus* eyecolor, about 18,000 flies were examined after EMS treatment of the isogenized $IcFRT^{2L2R}$#60 (as shown in Table 3). 4 flies, EMS1 to 4, were selected.

Table 3. Germline clonal analysis of isogenized $cFRT^{2L2R}$ chromosome

	MO %		Hatching rate in GLC		DE%	
IcFRT$^{#60}$-2L	156/184	0.85%	493/595	0.83%	5/595	0.0084%
IcFRT$^{#60}$-2R	153/178	0.86%	436/450	0.97%	3/450	0.0067%
IcFRT$^{#61}$-2L	148/162	0.91%	563/600	0.94%	6/600	0.0100%
IcFRT$^{#61}$-2R	132/158	0.84%	417/430	0.97%	3/430	0.0070%

MO %: mosaic ovary (NO. of developed ovary versus total ovary).
Hatch rate in GLC: NO. of hatched eggs/total eggs laid.
DE %: NO. of dead embryos/total eggs in germline clone analysis

[0099]   EMS1 had cream-colored eyes and the others with white and red mosaic eyecolors. After these 4 males were crossed with *w/w; Sco/CyO* females, different results for these 4 chromosomes were found. All the progeny of EMS1 had red eyes. EMS3 turned out to be male sterile. The progeny of EMS2 and EMS4 had either red eyes or white eyes with the ratio of 1:1. One red-eyed male and 5 white-eyed males from the EMS2 progeny and one white-eyed male from the EMS4 progeny were collected for further analyses. These putative $cFRT^{2R2LW}$ chromosomes were denominated NR2-1, NW2-1 to NW2-5 and NW4, respectively.

[00100]   To examine the recombination capacities of these modified chromosomes, germline clone analysis is generated. It turned out that the hatching rate of each strain was similar to the original untreated strain $IcFRT^{2L2R}$#60 (as shown in Table 4), and no particular embryonic phenotype was found.

Table 4. Hatching rate in GLC of $cFRT^{2L2RW}$ chromosomes

	GLC tested	Hatching rate in GLC
$IcFRT^{60}$	2L	493/595=82.9%
	2R	436/450=96.9%
NR2-1	2L	243/268=90.7%
	2R	208/227=91.6%
NW2-1	2L	178/200=89.0%
	2R	160/192=83.3%
NW2-2	2L	168/189=88.9%
	2R	160/175=91.4%
NW2-3	2L	120/141=85.1%
	2R	215/227=94.7%
NW2-4	2L	159/179=88.8%
	2R	159/187=85.0%
NW2-5	2L	131/155=84.5%
NW4	2L	211/219=96.3%
	2R	173/185=93.5%

[00101] After isogenization, it was confirmed that the chromosome behaved as a wild type chromosome (as shown in Table 3). This $cFRT^{2L2R}$ chromosome was used for a pioneer screen for the searching of essential loci on the second chromosome with specific maternal effects.

[00102] The failure to remove the miniwhite marker enforced us to use P[ry+] transposon as the mutagen. Two schemes described below were designed.

A.

1. +/Y; SM1/Sco; ry/ry X P[lArB]/ P[lArB]

2. P[lArB]/Y; SM1/+; ry/+ X w/w; cFRT/cFRT

3. yw/Y; Sco/CyO, Δ2-3; MKRS/+X P[lArB]/w; cFRT/SM1; +/+

4. _P[lArB]/Y; cFRT/CyO, Δ2-3; MKRS/+_ X _SM1/Sco;ry/ry_

5. look for (+/Y; _cFRT/SM1 or Sco; MKRS/ry_) males with _ry_+ eyecolor

6. (+/Y; _cFRT/SM1 or Sco; MKRS/ry_) X _SM1/Sco;ry/ry_

    a. if all _Sb_ flies are with _ry_$^+$ eyecolor, the P transposon may be on the MKRS chromosome

    b. if some _Sb_ and some _Sb_+ flies are with _ry_$^+$ eyecolor, the P transposon may be on the cFRT or the fourth chromosome.

    c. if all _Sb_ and all _Sb_+ flies are with _ry_$^+$ eyecolor, there may be multiple insertions on autosomes.

7. Select P transposon on _cFRT_ chromosome and determine their homozygous lethality.

B.

1. _P[lArB]/ P[lArB]; cFRT/cFRT; ry/ry_ X _yw/Y; Sco/CyO, Δ2-3; MKRS/+_

2. _P[lArB]/Y; cFRT^{2L2R} /CyO, Δ2-3; MKRS/+_ X _+/+; Sco/CyO; ry/ry_

3. Look for (+/Y; _cFRT^{2L2R} / CyO or Sco; MKRS/ry_) males with _ry_+ eyecolor

4. (+/Y; _cFRT/SM1 or Sco; MKRS/ry_) with _ry_+ eyecolor X +/+; _Sco/CyO; ry/ry_

5. examine the location of _P[lArB]_ insertions as that described in A. scheme.

[00103]   Several different _P[lArB]_ insertions on X chromosome were tested for their transposition efficiency. _P[lArB]_ insertions with normal efficiency (Spradling et al., 1999) of transposition will be candidates for future large scale screening. The _P_ transposon on X chromosome was used as the mutagen, determined whether the _P_-induced mutations on the second chromosome were

created by the disruption of essential genes causing the homozygous lethality, analyzed the possible maternal functions based on the FLP-DFS technique (Chou and Perrimon, 1996), and recovered genomic DNA segments flanking P insertions by plasmid rescue and inversed PCR methods.

[00104] From 189 lines with P insertions on the $cFRT^{2L2R}$ chromosome, 36 homozygous lethal lines are obtained. The 19 % homozygous lethal rate is compatible with previous P-induced homozygous rate (Spradling et al., 1999). This further demonstrated that the $cFRT^{2L2R}$ can perform normal as a wild type chromosome for P transposition. As shown in Table 5, the four classes of zygotic lethals with specific maternal effect phenotypes (Perrimon et al., 1989): germ cell lethal (GCL) phenotype, abnormal oogenesis (AO) phenotype, maternal effect (ME) and maternal effect rescuable (MER) and no maternal effect (NME) phenotype can be assigned to the essential loci on the $cFRT^{2L2R}$ chromosome.

Table 5. Result of the primary screen for essential loci with specific maternal effects using *the cFRT2L2R* chromosome

Stock #	2L	2R	Phenotype	Gene	Function	Allele

Germ Cell Lethal phenotype

Stock #	2L	2R	Phenotype	Gene	Function	Allele
1168c2	no eggs	NME	arrested at stage2	CG13790		New
				CG13791		New
a150a	no eggs	NME	arrested at stage2	AC3	Guanylate cyclases	New
				CG1512	cell cycle regulator (cullin)	New
a138	no eggs		arrested at stage2	CycE	cell cycle regulator (cyclin)	Known Known
R069	no eggs	NME	arrested at stage2	CG9586/Cg		New

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1168-2	no eggs	NME	arrested at stage2			ND
a137	NME	no eggs	arrested at stage2			ND
1168e	NME	no eggs	arrested at stage2			ND

Abnormal Oogenesis phenotype

				BcDNA:GM		
1164f2	AO	NME	arrested at stage6	14618	translation factor	New
				CG14040	transporter	New
b26b	NME	AO	arrested at stage6	Sin3A	transcription factor	Known
b235	NME	AO	arrested at stage6			ND
F263	NME	AO	arrested at stage6	beta-tub56D		New
F284	NME	AO	arrested at stage7	CG11508	snRNA	New
594-28	NME	AO	arrested at stage7	CG12864	motor protein	New

Embryo with specific Maternal Effect phenotype

				CG17280/2	Cytochrome C	
Y016-1	NME	MER	posterior group	R	subunit	New
				Or45b-		
b54a	NME	MER	posterior group	CG12931	Olfactory receptor	New
				CG1888	NLS BP	New
			denticle belts		enzyme (RNA Pol	
b347	MER	NME	fusion	CG10685	1)	New
b241b	ME	NME	dorsalized	dorsal	transcription factor	Known
			denticle belts			
a210a	MER	NME	fusion	Out at first		Known
				bratCG1071		
			like gap gene	9	transcription factor	Known
					transmembrane	
4799	NME	MER	lawn-of-denticles	tout-velu	protein	Known
			denticle belts			
b345	ME	NME	fusion			ND
			(like kismet)			
a350	NME	MER	no phenotype			ND

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stock #	2L	2R	phenotype	gene	function	allele

No maternal effect phenotype

stock #	2L	2R	phenotype	gene	function	allele
a22	NME			*CG12052*	transcription factor	New
b181	NME	NME	denticle fusion	*kismet*		Known
1168f1	NME	NME				ND
1164b	NME	NME				ND
594c1	NME	NME		.		ND
1164	NME	NME				ND
1164c4	NME	NME				ND
1164a13	NME	NME				ND
a194	NME	NME				ND
a256	NME	NME				ND
a172b	NME	NME				ND
a301	NME	NME				ND
b187	NME	NME				ND
b140b	NME	NME				ND
b30a	NME	NME				ND

Germ cell lethal phenotype is assigned if no eggs is laid for more than 15 GLC females examined.

AO: abnormal oogenesis; MER: paternal rescuable maternal effect; ME: strict maternal effect

NME: no maternal effect; ND: molecular properties not yet determined. When there is no known alleles in FlyBase databank, the *P* insertion is said to be assigned as a new allele.

[00105]   Either left or right arms can be the target of the *P* transposition and be analyzed for their germline clone phenotype.   This is the first time to demonstrate that a double *FRT* chromosome can be used for GLC analysis for both arms simultaneously after *P* mutagenesis.

[00106] The molecular nature of these essential loci were immediately determined. From mutation lines analyzed, both new and known alleles were recovered. Known genes with specific GLC embryonic phenotypes were recovered. By complementation test and DNA sequences comparison, known genes with compatible phentotypical descriptions, for example, *dorsal* (*b241b*), *tout-velu* (*4799*) and *kismet* (*b181*), were confirmed both molecularly and genetically. These cases demonstrated that the mutation phenotypes obtained were resulted from the disruption of essential loci by *P* insertions instead of the presentation of unknown endogenous chromosomal denaturation created during the process of establishing the $cFRT^{2L2R}$ chromosome.

[00107] This preliminary result clearly demonstrates that the $cFRT^{2L2R}$ chromosome will be feasible for the a large scale screen using the *P*-transposon as the mutagen to disrupt 95% essential loci on the *Drosophila* second chromosome.

[00108] While the invention has been described in terms of what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention needs not be limited to the disclosed embodiments. On the contrary, it is intended to cover various modifications and similar arrangements included within the spirit and scope of the appended claims which are to be accorded with the broadest interpretation so as to encompass all such modifications and similar structures.